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Heterochronic Phosphorelay Gene Expression as a Source of Heterogeneity in *Bacillus subtilis* Spore Formation▽†

Imke G. de Jong, Jan-Willem Veening, and Oscar P. Kuipers*

Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, Kluyver Centre for Genomics of Industrial Fermentation, University of Groningen, Haren, Netherlands

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In response to limiting nutrient sources and cell density signals, *Bacillus subtilis* can differentiate and form highly resistant endospores. Initiation of spore development is governed by the master regulator Spo0A, which is activated by phosphorylation via a multicomponent phosphorelay. Interestingly, only part of a clonal population will enter this developmental pathway, a phenomenon known as sporulation bistability or sporulation heterogeneity. How sporulation heterogeneity is established is largely unknown. To investigate the origins of sporulation heterogeneity, we constructed promoter-green fluorescent protein (GFP) fusions to the main phosphorelay genes and perturbed their expression levels. Using time-lapse fluorescence microscopy and flow cytometry, we showed that expression of the phosphorelay genes is distributed in a unimodal manner. However, single-cell trajectories revealed that phosphorelay gene expression is highly dynamic or "heterochronic" between individual cells and that stochasticity of phosphorelay gene transcription might be an important regulatory mechanism for sporulation heterogeneity. Furthermore, we showed that artificial induction or depletion of the phosphorelay phosphate flow results in loss of sporulation heterogeneity. Our data suggest that sporulation heterogeneity originates from highly dynamic and variable gene activity of the phosphorelay components, resulting in large cell-to-cell variability with regard to phosphate input into the system. These transcriptional and posttranslational differences in phosphorelay activity appear to be sufficient to generate a heterogeneous sporulation signal without the need of the positive-feedback loop established by the sigma factor SigH.

When nutrient sources are dwindling, the Gram-positive bacterium *Bacillus subtilis* can utilize a number of adaptive phenotypes such as the secretion of proteases and the development of genetic competence (for a recent review, see reference 49). The most sophisticated survival strategy that *B. subtilis* employs is the formation of a highly resistant endospore (34). Initiation of spore formation is governed by a complex multicomponent phosphorelay (Fig. 1) including the primary kinases KinA and KinB and two intermediate phosphotransferases, Spo0B and Spo0F (33). The phosphorelay activates Spo0A, the master sporulation regulator of sporulation, by phosphorylation (4), upon which phosphorylated Spo0A (Spo0A~P) can directly regulate more than 100 genes (29). This induces a chain of events that takes several hours to complete and involves the activation of a set of alternative sigma factors that give unidirectionality to the cascade, culminating in the formation of the endospore (10).

Interestingly, within isogenic populations of *B. subtilis* grown under identical conditions, not all cells enter this timely and costly developmental pathway. This type of phenotypic bifurcation is known as sporulation bistability or sporulation heterogeneity (9, 38). How populations of genetically identical cells bifurcate into phenotypically distinct subpopulations in the same environment is an important question for developmental biology. Competence development is another example of phenotypic bistability in *B. subtilis* (38). Positive feedback of the master competence regulator ComK on its own expression has been shown to be essential and sufficient for competence bistability (28, 37). For the initiation of sporulation, the origins of its heterogeneity seem to be more complex: although Spo0A~P also activates its own transcription, this positive-feedback loop is not essential for heterogeneous initiation of sporulation (50). Several lines of investigation indicate that the activity of the phosphorelay (the phosphorelay phosphate charge) determines the fraction of cells that commit to spore formation (8, 14, 15, 46, 50). However, whether sporulation heterogeneity originates from cell-to-cell variation in the phosphate charge and/or expression levels of the phosphorelay components is unknown. Another major question is whether sporulation heterogeneity originates from one or several (and if so, which) genes/proteins in the system. To address these questions, we constructed promoter-green fluorescent protein (GFP) fusions of the genes coding for the core phosphorelay components and P_{sigH} so that their expression could be followed in real time at the single-cell level. The expression of each individual phosphorelay gene was correlated with sporulation, and the impact of phosphorelay gene expression on sporulation heterogeneity was tested by systematically perturbing the system using knockouts and overproduction strains.

With time-lapse microscopy and flow cytometry experiments, we show that expression of the phosphorelay genes is distributed not bimodally but unimodally within the popula-

* Corresponding author. Mailing address: Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, Kluyver Centre for Genomics of Industrial Fermentation, University of Groningen, Haren, Netherlands. Phone: 31 (0) 50 363 2093. Fax: 31 (0) 50 363 2348. E-mail: o.p.kuipers@rug.nl.

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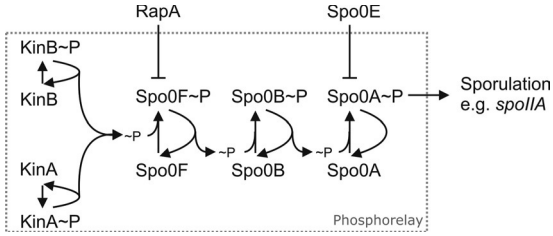

FIG. 1. Main phosphotransfer routes within the *Bacillus subtilis* phosphorelay.

tion. However, transcription of several phosphorelay genes appears to be dynamic and variable in time (heterochronic), and we found a correlation between the temporal gene activation and whether a cell sporulates or not. In addition, we provide data suggesting that these characteristics of phosphorelay gene expression result in cell-to-cell variability of the phosphorelay phosphate flow and thus sporulation heterogeneity. Finally, our results indicate that the fluctuations in the complex sporulation signal transduction cascade are sufficient for establishing a heterogeneous sporulation signal, without the need for specific positive-feedback loops.

MATERIALS AND METHODS

Oligonucleotides, plasmids, strains, and media. Oligonucleotides, plasmids and bacterial strains are listed in Tables 1, 2, and 3, respectively. *Bacillus subtilis* 168 trp+ was grown in TY, TLM, or 15% CDM at 37°C or 30°C (see below). *Escherichia coli* DH5α and MC1061 were used as hosts for cloning and grown in TY at 37°C. When required, the growth media were supplemented with antibiotics at the following concentrations: 5 μg ml^{-1} of chloramphenicol, 5 μg ml^{-1} of kanamycin, 100 μg ml^{-1} of spectinomycin, 4 μg ml^{-1} of erythromycin (*B. subtilis*), 150 μg ml^{-1} of erythromycin (*E. coli*), and 100 μg ml^{-1} of ampicillin (*E. coli*). Agar at 1.5% was included for solid medium.

Recombinant DNA techniques and oligonucleotides. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described before (36). Restriction enzymes were obtained from Roche (Mannheim, Germany), and all PCRs were performed with Phusion (NEB, United Kingdom), unless stated otherwise. Oligonucleotides

TABLE 1. Oligonucleotides used

Oligonucleotide	Sequence (5′→3′)
PsigH-F+HindIII	CGCGCAAGCTTGGCGGAATATCAATCGTATACAGG
PsigH-R+EcoRI+RBS	CGGCGGAATTCCATTTCCTCTCCTCCTCACGTAGATAGAAATATTATACAGTATTGG
PkinA-F+HindIII	CGCAAGCTTGGCGATTGAACATAGGAAACC
PkinA-R+EcoRI+RBS	GCGGAATTCCATTTCCTCTCCTCCTATGATCATCGTGTTTCGACATATACAG
PkinB-F+HindIII	CGCGCAAGCTTCATTTAATATCGCCGGATTGC
PkinB-R+EcoRI+RBS	CGGCGGAATTCCATTTCCTCTCCTCCTCGTATAAAATATGAATCTATTATAACAC
Pspo0F-F+HindIII	CGCGCAAGCTTCCGCACTGTCTTTGGCTGCG
Pspo0F-R+EcoRI+RBS	CGGCGGAATTCCATTTCCTCTCCTCCTATGATTTTCGTCAAAAGTAAGCAGTATTG
Pspo0B-F+HindIII	CGCGCAAGCTTCAAAGAACGGACGTGACTCTG
Pspo0B-R+EcoRI+RBS	CGGCGGAATTCCATTTCCTCTCCTCCTATTTAATTTCTTATTTAGGAGTCTGTATAAG
Pspo0A-F+HindIII	CGCAAGCTTCCGATCCAAGACTGTTGAAAG
Pspo0A-R+EcoRI+RBS	GCGGAATTCCATTTCCTCTCCTCCTATGTAGTTAACAGGATTCACCCTTGCTAC
PspoIIA-F+HindIII	CGCCCAAGCTTGAAAGAAACGCGCAAGTGAC
PspoIIA-R+PstI+RBS	CGGCGCTGCAGCATTTCCTCTCCTCCTTATGATCGGATAATGAGTGTTTCG
PrapA-F+HindIII	CGCAAGCTTGCGGTACGACAAACTATCCCGAAG
PrapA-R+EcoRI+RBS	GCGGAATTCCATTTCCTCTCCTCCTAATTACCCGAGATATGTCATATTTTATC
PkinA-F+KpnI+EcoRI	GCGGCGGTACCCGCGGCGAATTCTATCGTGTTCAGCCTAGAATC
PkinA-R+NheI	GCGGCGCTAGCCACAGAATCCCTCCTTTGCATG
sigH-F+SalI+RBS	GCACTGGTCGACTAGGAGGAGAGGAAATGAATCTACAGAACAACAAGGG
sigH-R+NheI + 2stop	GGGCATGCTAGCCTATTACAAACTGATTTCGCG
kinA-F+SalI+RBS	GCACTGGTCGACTAGGAGGAGAGGAAATGGAACAGGATACGCAGCATGTTAAACCACTTC
kinA-R+NheI	GGGCATGCTAGCCGCTGAGAGAAGGCCTAGAC
kinB-F+SalI+RBS	GCACTGGTCGACTAGGAGGAGAGGAAATGGAAATTCTAAAAGACTATCTTCTGC
kinB-R+NheI + 2stop	GGGCATGCTAGCTTACTAGTGAGGAAGATCAGCGGG
spo0F-F+SalI+RBS	GCACTGGTCGACTAGGAGGAGAGGAAATGATGAATGAAAAAATTTTAATCGTTG
spo0F-R+NheI + 2stop	GGGCATGCTAGCTTATCAGTTAGACTTCAGGGGCAGATATTTTTTGACG
spo0B-F+SalI+RBS	GCACTGGTCGACTAGGAGGAGAGGAAATGAAGGATGTTTCAAAAAATCAAGAAG
spo0B-R+NheI + 2stop	GGGCATGCTAGCTTACTAGTCCAACCCAATTTCAATCAGAC
spo0E-F+SalI+RBS	GCACTGGTCGACTAGGAGGAGAGGAAATGGGCGGTTCTTCTGAACAAG
spo0E-R+NheI	GGGCATGCTAGCGTAATGAGAATCGCCAGTTCC
kinA-F+SpeI+RBS	GCACTGACTAGTTAGGAGGAGAGGAAATGGAACAGGATACGCAGCATGTTAAACCACTTC
kinA-R+BamHI	GGGCATGGATCCCGCTGAGAGAAGGCCTAGAC
sigH_up-F	CGCTTCTGGGCTACCTTTTTCTAGAG
sigH_up-R+PstI	CGCGCTGCAGTCCGATCCCCCCGGCGCACGTAGATAG
sigH_down-F+XbaI	GCGCTCTAGATAATAGGAATTTATGCTATATTGACAG
sigH_down-R	CGGAAAGCCACTTCAACTGTAGCGTCG
kinA_up-F	CGTGATTACTGGATCGCAGCTC
kinA_up-R+PstI	CGCGCTGCAGAGAATCCCTCCTTTGCATGATCATCG
kinA_down-F+XbaI	GCGCTCTAGATAAAAACAACGGCTTAAACGCCG
kinA_down-R	GCTGATGGGAGTGAGGCACTGATG
kinB_up-F	CCTAATTCCGTGCTGTACTC
kinB_up-R+XbaI	GGCGCTCTAGATCGTGTGAAATCCTTTCGTATAAAATATG
kinB_down-F+EcoRI	CGGCGGAATTCCAAATCGATTGGAACTTTAATAGAATAAATG
kinB_down-R	GGGCTGGATACTGAAATTGC

TABLE 2. Plasmids

Plasmid	Genotype	Reference or source
pSG1151	*bla cat gfpmut1*	25
pJWV012	*bla sacA* P_{spoIIA}-mCherry Kan^r	48
pDR110	Amp^r Sp^r *lacI 'amyE amyE'* P_{spank}	Gift from D. Rudner
pAX01	*bla* Amp^r Erm^r *'lacA lacA'* P_{xyl} *xylR*	18
pBEST501	*bla* P_{repU}-*neo*	21
pVK71	*bla neo*::Sp	6
pGFP-*sigH*	*bla cat* P_{sigH}-GFP Cm^r	This study
pGFP-*kinA*	*bla cat* P_{kinA}-GFP Cm^r	This study
pGFP-*kinB*	*bla cat* P_{kinB}-GFP Cm^r	This study
pGFP-*spo0F*	*bla cat* P_{spo0F}-GFP Cm^r	This study
pGFP-*spo0B*	*bla cat* P_{spo0B}-GFP Cm^r	This study
pGFP-*spo0A*	*bla cat* P_{spo0A}-GFP Cm^r	This study
pGFP-*spoIIA*	*bla cat* P_{spoIIA}-GFP Cm^r	This study
pGFP-*rapA*	*bla cat* P_{rapA}-GFP Cm^r	This study
pmCherry-*kinA_sacA*	*bla sacA* P_{kinA}-mCherry Kan^r	This study
pDR110-*sigH*	Amp^r Sp^r *lacI 'amyE amyE'* P_{spank}-*sigH*	This study
pDR110-*kinA*	Amp^r Sp^r *lacI 'amyE amyE'* P_{spank}-*kinA*	This study
pDR110-*kinB*	Amp^r Sp^r *lacI 'amyE amyE'* P_{spank}-*kinB*	This study
pDR110-*spo0F*	Amp^r Sp^r *lacI 'amyE amyE'* P_{spank}-*spo0F*	This study
pDR110-*spo0B*	Amp^r Sp^r *lacI'amyE amyE'* P_{spank}-*spo0B*	This study
pDR110-*spo0E*	Amp^r Sp^r *lacI'amyE amyE'* P_{spank}-*spo0E*	This study
pAX01-*kinA*	*bla* Amp^r Erm^r *'lacA lacA'* P_{xyl}-*kinA xylR*	This study

were purchased from Biolegio (Nijmegen, Netherlands). All constructs were sequence verified. *B. subtilis* was transformed as described before (19).

Time-lapse fluorescence microscopy. Overnight cultures were inoculated from −80°C stocks and grown at 30°C in TLM [62 mM K_2HPO_4, 44 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 6.5 mM sodium citrate, 0.8 mM $MgSO_4$, 0.02% Casamino Acids, 27.8 mM glucose, 0.1 mM and L-tryptophan, with the pH set to 7 using a KOH solution]. The following morning, the cells were diluted 1:10 in 15% prewarmed CDM [62 mM K_2HPO_4, 44 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 6.5 mM sodium citrate, 0.8 mM $MgSO_4$, 2.2 mM glucose, 2.1 mM L-glutamic acid, 6 μM L-tryptophan, 7.5 μM $MnCl_2$, 0.15× metal mix (45)] and grown for 4 h at 30°C. Single cells (rediluted in 15% CDM) were loaded on 15% CDM supplemented with 1.5% high-resolution agarose (Sigma) (for detailed information, see reference (50) and, if relevant, 100 μM isopropyl-β-D-1-thiogalactopyranoside (IPTG) or 0.5% xylose (for induction of transcription from the P_{spank} or P_{xyl} promoter, respectively). The outgrowth of a single cell into a microcolony monolayer was achieved by growing the cells in an environmental chamber at 30°C. The following equipment/settings were used for time-lapse microscopy (provided by DeltaVision, United Kingdom): IX71 microscope (Olympus), CoolSNAP HQ2 camera (Princeton Instruments), 300-W xenon light source, 60× bright-field objective (1.25 numerical aperture [NA]), GFP filter set (Chroma; excitation at 470/40 nm and emission at 525/50 nm), mCherry filter set (Chroma; excitation at 572/35 nm and emission at 632/60 nm). Snapshots for movies were taken at intervals of 8 or 12 min using 10% APLLC white LED light and a 0.05-s exposure for bright-field pictures, 10% xenon light and a 0.5-s exposure for GFP detection (unless indicated otherwise), and 32% Xenon light and a 0.8-s exposure for mCherry detection. Raw data were stored using softWoRx 3.6.0 (Applied Presicion) and adjusted for publication using Adobe Photoshop 5.0 (Adobe), ImageJ (http://rsbweb.nih.gov/ij/), and CorelDRAW X3 (Corel Corporation).

Flow cytometry. Cultures were inoculated from −80°C stocks and grown overnight at 37°C in TY medium supplemented with chloramphenicol. After dilution to an optical density at 600 nm (OD_{600}) of ~0.1, cells were grown for 2 h and transcription of the inducible genes was induced with 100 μM IPTG and/or 0.5% xylose at the beginning of the exponential phase. Samples for flow analysis were taken every hour, and GFP levels of at least 20,000 cells were measured with a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, Netherlands) using an argon laser (488 nm). Raw data were taken using EXPO32 software (Beckman Coulter). WinMDI 2.9 was used for data analysis (http://en.bio-soft.net/other/WinMDI.html).

Plasmid construction. (i) Promoter-reporter fusion plasmids. To construct the promoter-GFP fusion plasmids, approximately 600 to 700 bp of the corresponding promoter regions, including all known regulatory sequences (according to http://dbtbs.hgc.jp/), was amplified by PCR using *B. subtilis* 168 chromosomal DNA as a template. For this study we only examined transcriptional regulation, and to obtain maximal sensitivity we replaced the native ribosome binding site (RBS) in all cases with a strong RBS using ATG as a start codon (47, 51). The PCR fragments were inserted in front of the *gfpmut1* gene present on plasmid pSG1151 (25) via the HindIII and EcoRI or HindIII and PstI restriction sites.

To construct pmCherry-*kinA_sacA*, approximately 600 bp of the *kinA* promoter region, including all known regulatory sequences (according to http://dbtbs.hgc.jp/), was amplified by PCR using *B. subtilis* 168 chromosomal DNA as a template. The PCR fragment was inserted in front of the mCherry gene present on plasmid pJWV012 (48) via the KpnI and NheI restriction sites.

(ii) Overexpression plasmids. Plasmid pDR110 (a kind gift of David Rudner) carrying the tight IPTG-inducible *spank* promoter was used as vector for the IPTG-inducible constructs (Table 2). Genes coding for the proteins to be overproduced were amplified by PCR using *B. subtilis* 168 chromosomal DNA as a template, and strong translation signals were inserted in the primer for all constructs. PCR fragments were cleaved with NheI and SalI and ligated into the corresponding sites of pDR110. To construct a xylose-inducible *kinA* overexpression plasmid, *kinA* was amplified by PCR as described above, whereby SpeI and BamHI were used as integration sites for ligation of the PCR product into pAX01.

Strain construction. (i) Promoter-reporter fusion strains. The promoter-GFP fusion plasmids were inserted into the native locus on the *B. subtilis* 168 trp+ chromosome by Campbell-type integration, leaving the wild-type background of the corresponding genes intact. Colonies were selected for chloramphenicol resistance, and correct integration was confirmed by PCR using a specific forward primer for each strain on the genome located upstream of the promoter region and pGFP-seq-R (5′GTTGGCCATGGAACAGGTAG3′) as a reverse primer for all constructs.

pmCherry-*kinA_sacA* was inserted into the *sacA* locus on the *B. subtilis* 168 trp+ chromosome of IDJ008, leaving the wild-type background of *kinA* intact. Colonies were selected for kanamycin resistance, and correct integration was checked by PCR.

(ii) Overexpression strains. The IPTG-inducible overexpression plasmids were inserted into the *B. subtilis* 168 trp+ chromosome by double crossover into the nonessential *amyE* locus. Colonies were selected on spectinomycin plates, and correct integration of the overproduction constructs was confirmed by lack of a halo on TY plates containing 1% starch. The xylose-inducible pAX01-*kinA* construct was inserted into the *B. subtilis* 168 trp+ chromosome by double crossover into the nonessential *lacA* locus. Colonies were selected on erythromycin plates, and correct integration of the overproduction constructs was confirmed by PCR.

(iii) Replacement mutants. Replacement mutants were obtained as follows. About 2,000 bp upstream (including the start codon) and downstream (excluding the stop codon) of the corresponding genes was amplified using *B. subtilis* 168 chromosomal DNA as a template. Corresponding primers are listed in Table 1.

TABLE 3. Strains

Species and strain	Relevant genotype	Reference or source
E. coli		
MC1061	*supE44 hsdR17 recA1 gyrA96 thi-1 relA1*	Laboratory stock
DH5α	F$^-$ *araD139* Δ(*ara-leu*)*7696* Δ(*lac*)*X74 galU galK hsdR2 mcrA mcrB1 rspL*	Laboratory stock
B. subtilis		
JWV002	Trp$^-$ *amyE*::P$_{spoIIA}$-mCherry Cm^r	48
168 trp+	Trp$^+$	SysMo consortium
Promoter-GFP strains[a]		
IDJ001	P$_{sigH}$-GFP Cm^r	This study
IDJ002	P$_{kinA}$-GFP Cm^r	This study
IDJ003	P$_{kinB}$-GFP Cm^r	This study
IDJ004	P$_{spo0F}$-GFP Cm^r	This study
IDJ005	P$_{spo0B}$-GFP Cm^r	This study
IDJ006	P$_{spo0A}$-GFP Cm^r	This study
IDJ007	P$_{spoIIA}$-GFP Cm^r	This study
IDJ008	P$_{rapA}$-GFP Cm^r	This study
Overexpression strains[a]		
IDJ010	*amyE*::P$_{spank}$-*sigH* Sp^r	This study
IDJ011	*amyE*::P$_{spank}$-*kinA* Sp^r	This study
IDJ012	*amyE*::P$_{spank}$-*kinB* Sp^r	This study
IDJ012	*amyE*::P$_{spank}$-*spo0F* Sp^r	This study
IDJ013	*amyE*::P$_{spank}$-*spo0B* Sp^r	This study
IDJ014	*amyE*::P$_{spank}$-*spo0E* Sp^r	This study
IDJ015	*lacA*::P$_{xyl}$-*kinA* Erm^r	This study
Gene replacement strains[a]		
IDJ016	*sigH*::*neo* Km^r	This study
IDJ017	*kinA*::*neo* Km^r	This study
IDJ018	*kinA*::*neo*::pVK71 Sp^r	This study
IDJ019	*kinB*::*neo* Km^r	This study
Combination strains[a]		
IDJ020	P$_{spoIIA}$-GFP Cm^r *amyE*::P$_{spank}$-*sigH* Sp^r	This study
IDJ021	P$_{spoIIA}$-GFP Cm^r *amyE*::P$_{spank}$-*kinA* Sp^r	This study
IDJ022	P$_{spoIIA}$-GFP Cm^r *amyE*::P$_{spank}$-*kinB* Sp^r	This study
IDJ023	P$_{spoIIA}$-GFP Cm^r *amyE*::P$_{spank}$-*spo0F* Sp^r	This study
IDJ024	P$_{spoIIA}$-GFP Cm^r *amyE*::P$_{spank}$-*spo0B* Sp^r	This study
IDJ025	P$_{spoIIA}$-GFP Cm^r *lacA*::P$_{xyl}$-*kinA* Erm^r	This study
IDJ026	P$_{spoIIA}$-GFP Cm^r *amyE*::P$_{spank}$-*sigH* Sp^r *lacA*::P$_{xyl}$-*kinA* Erm^r	This study
IDJ027	P$_{spoIIA}$-GFP Cm^r *amyE*::P$_{spank}$-*spo0E* Sp^r *lacA*::P$_{xyl}$-*kinA* Erm^r	This study
IDJ028	P$_{sigH}$-GFP Cm^r *amyE*::P$_{spank}$-*kinA* Sp^r	This study
IDJ029	P$_{kinA}$-GFP Cm^r *amyE*::P$_{spank}$-*kinA* Sp^r	This study
IDJ030	P$_{kinB}$-GFP Cm^r *amyE*::P$_{spank}$-*kinA* Sp^r	This study
IDJ031	P$_{spo0F}$-GFP Cm^r *amyE*::P$_{spank}$-*kinA* Sp^r	This study
IDJ032	P$_{spo0B}$-GFP Cm^r *amyE*::P$_{spank}$-*kinA* Sp^r	This study
IDJ033	P$_{spo0A}$-GFP Cm^r *amyE*::P$_{spank}$-*kinA* Sp^r	This study
IDJ034	P$_{spoIIA}$-GFP Cm^r *sigH*::*neo* Km^r	This study
IDJ035	P$_{spoIIA}$-GFP Cm^r *kinA*::*neo* Km^r	This study
IDJ036	P$_{spoIIA}$-GFP Cm^r *kinB*::*neo* Km^r	This study
IDJ037	P$_{spoIIA}$-GFP Cm^r *kinA*::*neo*::Sp Sp^r *kinB*::*neo* Km^r	This study
IDJ038	P$_{spoIIA}$-GFP Cm^r *amyE*::P$_{spank}$-*kinA* Sp^r *sigH*::*neo* Km^r	This study
IDJ039	P$_{rapA}$-GFP Cm^r P$_{kinA}$-mCherry Km^r	This study

[a] All strains are derivatives of 168 trp+.

The *neo* cassette was taken from pBEST501 (21) using PstI and XbaI or XbaI and EcoRI, respectively. The three fragments (upstream region, *neo* cassette, and downstream region) were ligated and directly transformed to *B. subtilis* strain JWV002 (48), which was used as an in-between host, before transformation to the target strain *B. subtilis* 168 trp+. Colonies were selected for kanamycin resistance, and correct chromosomal integration was verified by PCR.

(iv) Strain combinations. Combinations of the strains were made by transforming a promoter-GFP strain with pmCherry-*kinA_sacA* or chromosomal DNA from the IPTG-inducible strains and/or knockouts. Transformants were selected for the antibiotic resistance marker, and correct integration was verified by PCR and/or lack of a halo on TY plates containing 1% starch.

RESULTS

The core phosphorelay components are unimodally expressed. The major sporulation kinases KinA and KinB autophosphorylate and transfer the phosphate to the phospho-

transferase Spo0F (4). Spo0B receives the phosphate from Spo0F~P, and Spo0B~P subsequently phosphorylates Spo0A (Fig. 1). In response to phosphorylation, Spo0A~P dimerizes and is able to bind to its recognition sequence, the so-called 0A box (26). Sporulation-specific promoters, such as the *spoIIA* promoter, are activated by high concentrations of Spo0A~P (14). Using bulk population-wide LacZ assays, it has been shown that transcription of the phosphorelay genes increases during the early stages of sporulation (see, e.g., reference 1). However, it is unknown how the phosphorelay genes are regulated at the single-cell level. Previously it was shown that not all cells within clonal *B. subtilis* populations activate the *spoIIA* promoter and consequently form spores (8, 46) (Fig. 2H and Fig. 3H; see Movie S8 in the supplemental material). The simplest explanation for the bimodal expression pattern of *spoIIA* is that the heterogeneity is already present earlier than expression of *spoIIA*, at the level of the individual phosphorelay components. To test this hypothesis, we constructed promoter-GFP fusions of the core phosphorelay components and P_{sigH} and examined their GFP expression at the single-cell level using time-lapse microscopy. Individual cells were grown directly under the microscope on top of a thin layer of agarose in such a way that single cells grew into sporulating microcolonies (see Materials and Methods). Bright-field and fluorescence images were acquired at periodic intervals to generate time-lapse movies which contain information encompassing the complete cell division history of individual cells, their GFP expression status, and the final cell fate decision (see Materials and Methods and reference 50). We focused on sporulation heterogeneity only. Other heterogeneous phenotypes (e.g., competence development or protease production) that might be displayed were not examined. For each promoter-GFP fusion, at least two independent experiments were performed, and at least two developing microcolonies were analyzed per experiment (thus there were minimally four movies per strain). Time-lapse microscopy revealed that the expression levels of *kinA*, *kinB*, *spo0F*, *spo0A*, and *sigH* (also known as *spo0H*) increased with time until spore formation occurred and that the activity of all genes exhibited a large cell-to-cell variability (see Movies S1 to S8 in the supplemental material). Single-cell analyses of the movies showed a unimodal, broad, long-tailed Gaussian distribution in gene expression for *kinA*, *spo0F*, and *sigH* (Fig. 2; see Fig. S1 in the supplemental material). When all cells from a single microcolony (80 to 120 cells) were analyzed, the fluorescence distribution for the reporter strains of *kinB* and *spo0A* appeared to be almost bimodal (Fig. 2). However, when the data for corresponding time points from multiple microcolonies are pooled, a broad unimodal distribution becomes apparent (Fig. 2, insets; see Fig. S1 in the supplemental material). Thus, we conclude that also in the case of *kinB* and *spo0A*, expression is unimodal. Interestingly, GFP from the *spo0B* promoter was expressed with a relatively small cell-to-cell variability (note the low GFP expression levels, close to the background fluorescence of the wild type), and in contrast to that of the other phosphorelay genes, *spo0B* expression did not seem to increase during microcolony development (Fig. 2F; see Fig. S1F and Movie S6 in the supplemental material).

Taken together, these data indicate that bimodal expression of *spoIIA* is not the result of bimodal expression of one of the phosphorelay components.

High expression of *kinA*, *spo0F*, and *spo0A* at early stages of microcolony development correlates with spore formation. Although the phosphorelay genes did not exhibit a bimodal expression pattern, their variability in expression levels might cause sporulation heterogeneity if the timing of their induction differs between cells. To test this, we used GFP reporters to follow the expression of genes encoding components of the phosphorelay in a number of lineages that resulted in either sporulating or nonsporulating cells as judged by bright-field microscopy after 20 h during microcolony development (see Movies S1 to S8 in the supplemental material). At least two independent experiments were performed, and a minimum of four sporulating and four nonsporulating lineages of each promoter-GFP strain (IDJ001 to IDJ007) were analyzed per independent experiment. Note that it is not possible to directly compare the timings of trajectories from different experiments, since not only does the time of adaptation to the agarose slide vary between individual cells, but also the time between the preparation of the microscopic slide and the start of the movie differs for each experiment. Nevertheless, the timings of GFP expression from trajectories of sporulating cells can be compared by approximating the time between a specific expression trend and actual spore formation. Single-cell trajectories of the phosphorelay reporters showed that cells that formed spores early during microcolony development showed higher expression of *kinA*, *spo0F*, and *spo0A* than their nonsporulating siblings at the same time point within the same microcolony (Fig. 3, red lines; see Fig. S2 and S3 [left panel] in the supplemental material). In some cases nonsporulating cells also showed a peak of *spo0F* expression simultaneously with sporulating cells, but never earlier (see Fig. S2 and S3 in the supplemental material). The peak in expression occurred approximately 250 min before the endospore became visible (indicated by the last red point plotted in each graph). Subsequently, the GFP signals expressed from P_{sigH}, P_{kinA}, P_{spo0F}, and P_{spo0A} dropped dramatically, probably because of activation of the late sporulation-specific sigma factors and/or deactivation of sigma A (13, 20, 27).

Interestingly, cells that did not form spores during the time course of the experiment often showed similar (*kinA* and *spo0A*) or even stronger (*sigH* and *spo0F*) promoter activity of the phosphorelay genes at later stages of microcolony development than their sporulating siblings within the same microcolony (Fig. 3, black lines; see Fig. S2 and S3 [right panel] in the supplemental material). It cannot be excluded that some cells which did not sporulate during the experiment would ultimately have formed spores later (compare, e.g., red lines in Fig. S3G in the supplemental material). In all experiments performed, no particular differences could be found in the *kinB* expression patterns of sporulating and nonsporulating cells. This might be due to a supporting, but not primary, function of KinB to feed phosphate into the system. However, expression of *kinB* as well as *sigH* increases with time, suggesting that they might be sensitive to very low levels of Spo0A~P and that their regulation differs from that of other phosphorelay genes. The other peculiar phosphorelay component is Spo0B. As mentioned above, its expression is low, and the cell-to-cell differences are too small to reveal variations in expression patterns of sporulating and nonsporulating cells (Fig. 3F; see Fig. S2F and S3F in the supplemental material).

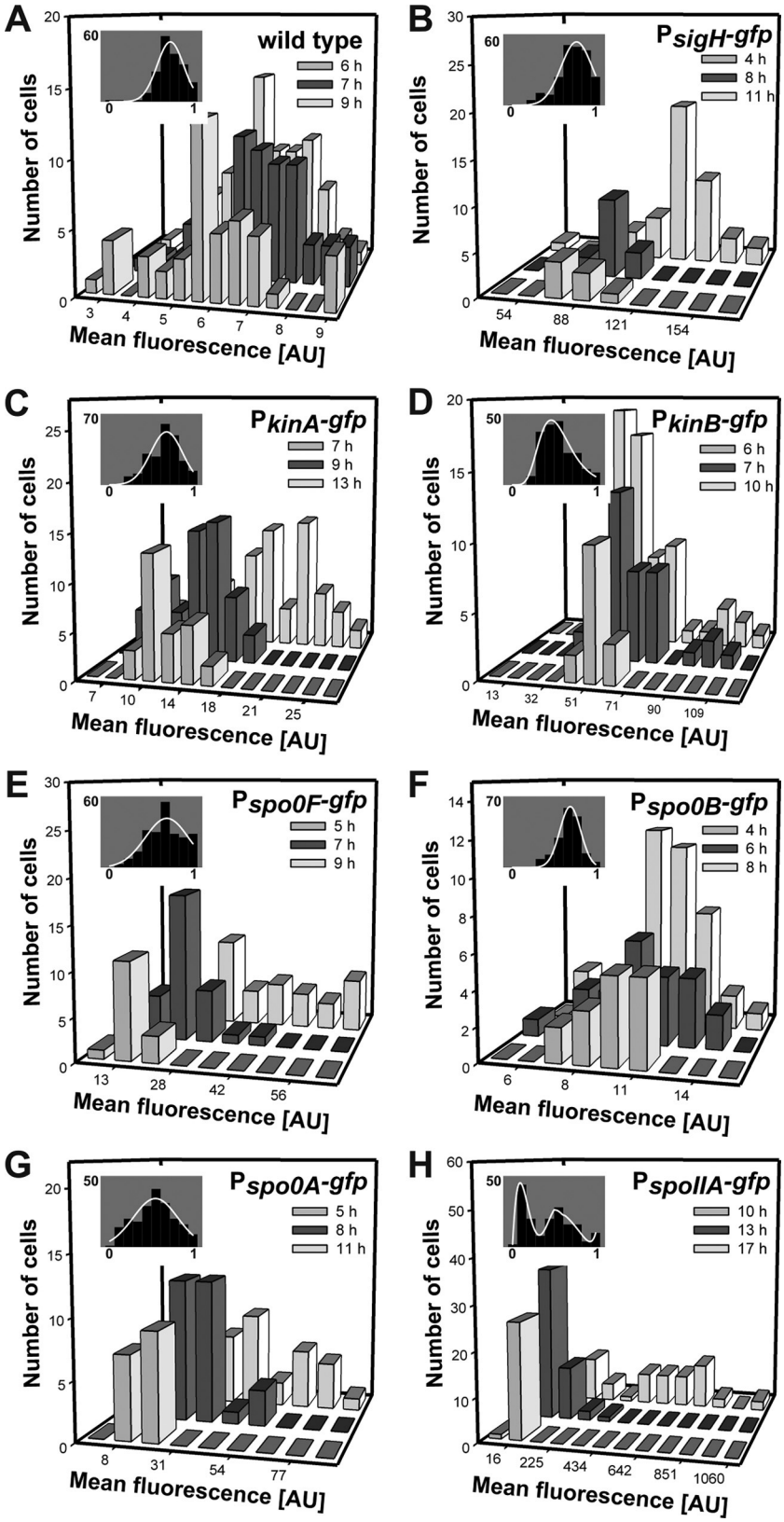

FIG. 2. Heterogeneous expression of the main phosphorelay components and *sigH*. Snapshots of early, middle, and late microcolony growth phases from time-lapse experiments (see Movies S1 to S8 in the supplemental material) were selected to create histograms displaying the distribution of GFP expression during microcolony development. (A) Wild type (168 trp+, not carrying a GFP construct); (B) P_{sigH}-GFP (IDJ001); (C) P_{kinA}-GFP (IDJ002); (D) P_{kinB}-GFP (IDJ003); (E) P_{spo0F}-GFP (IDJ004); (F) P_{spo0B}-GFP (IDJ005); (G) P_{spo0A}-GFP (IDJ006); (H) P_{spoIIA}-GFP (IDJ007). The insets display normalized data (late time point) from cells of at least three colonies per strain (black bars). For all strains except the P_{spoIIA}-GFP mutant, a Gaussian-like curve could be fitted (white line). The complete data set is shown in Fig. S1 in the supplemental material.

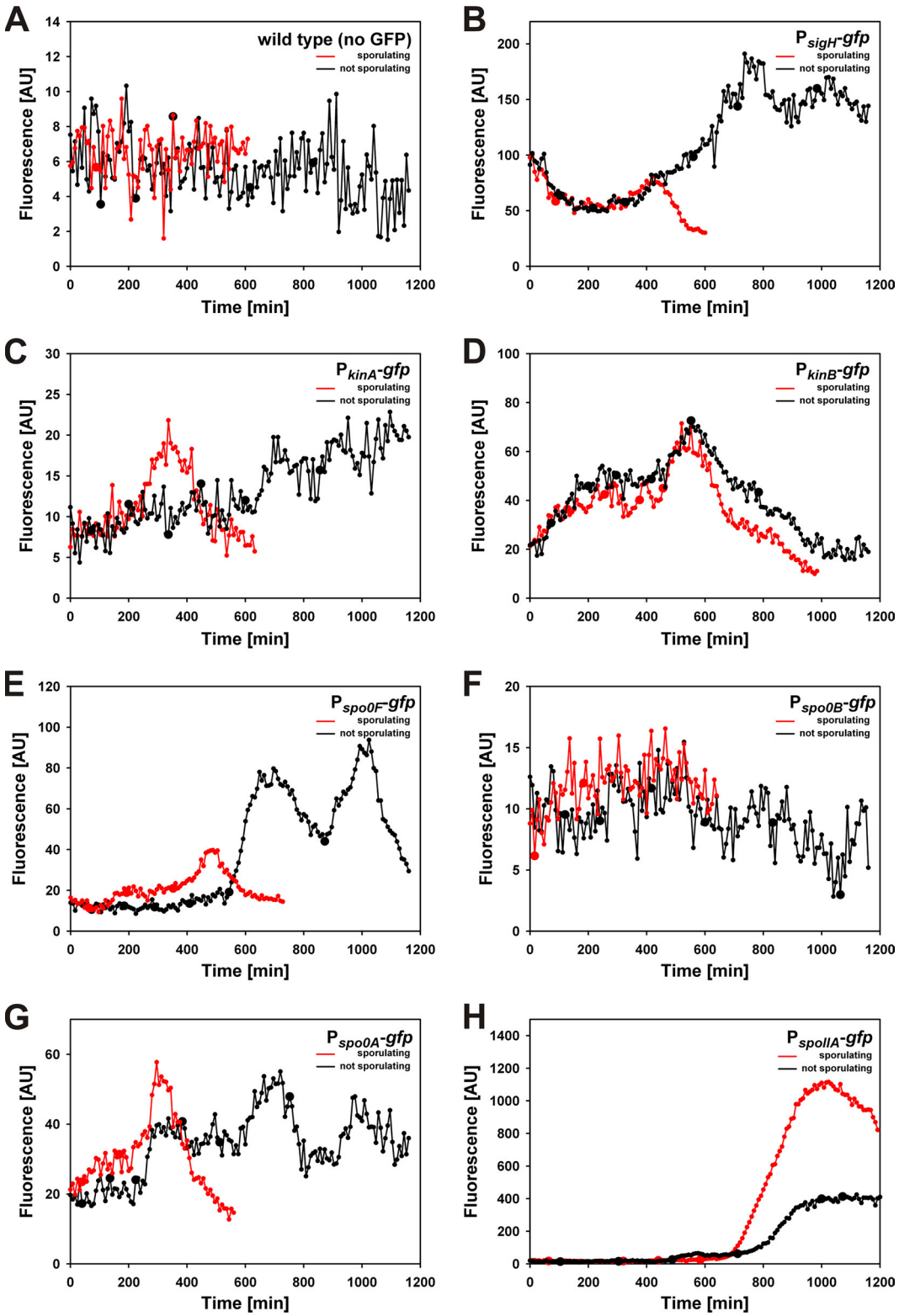

FIG. 3. Dynamic expression of the main phosphorelay components and *sigH*. GFP expression from randomly selected cells was followed through time by time-lapse microscopy (see Movies S1 to S8 in the supplemental material), resulting in single-cell trajectories of separate lineages, each leading to a sporulating (red lines) and a nonsporulating cell (black lines). After cell division, indicated by an increase in symbol size on the trace, one of the two resulting siblings was arbitrarily selected for further analysis. The traces were stopped when a spore became visible in the corresponding movie. (A) Wild type (168 trp+, not carrying a GFP construct); (B) P_{sigH}-GFP (IDJ001); (C) P_{kinA}-GFP (IDJ002); (D) P_{kinB}-GFP (IDJ003); (E) P_{spo0F}-GFP (IDJ004); (F) P_{spo0B}-GFP (IDJ005); (G) P_{spo0A}-GFP (IDJ006); (H) P_{spoIIA}-GFP (IDJ007).

These data support the hypothesis that the timing of phosphorelay gene expression, or at least of *kinA*, *spo0F*, and *spo0A* expression, is important in determining whether a cell will initiate sporulation or not.

Nonsporulating cells display high *rapA* transcription levels. As shown in Fig. 3 and in Fig. S2 and S3 in the supplemental material, early activation of at least *kinA*, *spo0F*, and *spo0A* seems to be important for successful spore formation. Strikingly, however, most nonsporulating cells exhibited relatively high phosphorelay transcription levels during late microcolony development but did not develop spores during the experimental time course. Thus, the question remains why these cells do not initiate sporulation despite relatively high levels of the phosphorelay components such as KinA. The most likely explanation for this paradoxical observation is that although the level of kinases is sufficient, the net activity of the phosphorelay is too low to obtain high levels of Spo0A~P. Phosphate groups can be drained from the phosphorelay by a number of phosphatases, including the major sporulation phosphatase RapA (33). Recently, it was shown that the levels of RapA are high in a subpopulation of cells during microcolony development (3). To assess the transcription levels of *rapA* and *kinA* simultaneously in single cells, we performed time-lapse microscopy experiments using a double-labeled strain in which P_{rapA} was fused to the GFP gene and P_{kinA} was fused to the mCherry gene. Information on the background fluorescence can be found in Fig. S4 in the supplemental material. Single-cell trajectories of the double-labeled strain IDJ039 show that mCherry transcription from P_{kinA} is more or less similar in both sporulating and nonsporulating cells, whereas GFP transcription from P_{rapA} is relatively low in sporulating cells, compared to high RapA transcription levels in cells that did not sporulate within the experimental time frame (Fig. 4A). The peak of mCherry expression from P_{kinA} is not as pronounced and is shifted to the right in time compared to the corresponding fusion to GFP shown in Fig. 3. These differences might be observed due to the limited brightness and longer maturation time of mCherry compared to GFP. To ensure that the difference in *rapA* expression is valid for all cells, all 61 cells from the microcolony were analyzed at a time point at which the first spore is not yet visible (786 min). This analysis shows that indeed a significant difference ($P < 0.01$ by Student's t test) in P_{rapA}-GFP expression levels exists between spore formers and nonsporulating cells (22 ± 15 versus 75 ± 28 arbitrary GFP units, respectively) (Fig. 4B).

Together, these data suggest that increased phosphatase expression prevents cells from sporulation, even if transcription of the phosphorelay genes is high.

KinA and KinB both contribute to sporulation heterogeneity. While the heterochronicity of *kinA*, *spo0F*, and *spo0A* gene expression is correlated with the initiation of sporulation (Fig. 2 and 3; see Fig. S1, S2, and S3 in the supplemental material), sporulation heterogeneity also seems to originate from cell-to-cell differences in the phosphate flow of the phosphorelay (8, 46) (Fig. 4). To test whether perturbations in the phosphate input have an effect on sporulation heterogeneity, we constructed strains with knockout or overproduction mutations of the genes coding for the sporulation kinases KinA and KinB. The corresponding mutations were introduced in a strain harboring the P_{spoIIA}-GFP reporter, and cells were monitored by fluorescence time-lapse microscopy and/or flow cytometry. As shown in Fig. 5A, spores in either a *kinA* (IDJ035) or *kinB* (IDJ036) single mutant were still readily formed. While fewer cells formed spores in either a *kinA* or *kinB* mutant back-

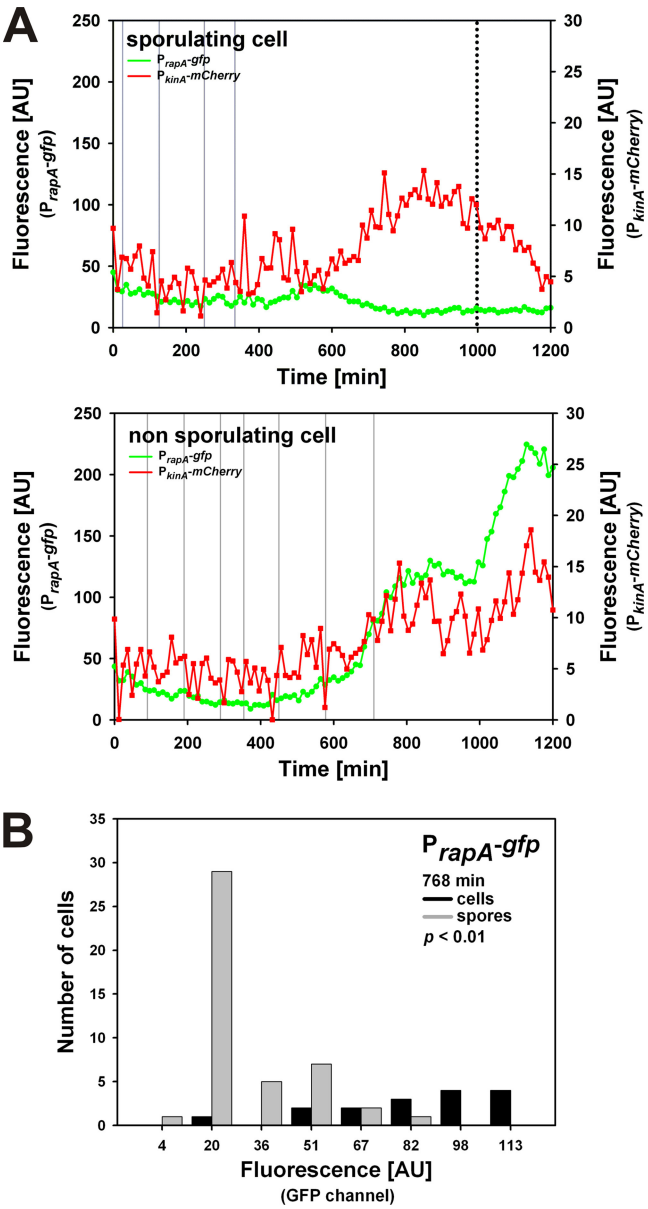

FIG. 4. Sporulation of cells is prevented by elevated levels of RapA. (A) GFP (green circles) and mCherry (red squares) production by IDJ039 (harboring P_{rapA}-GFP and P_{kinA}-mCherry) was followed through time during microcolony development (see Movie S9 in the supplemental material). After each cell division, indicated by gray vertical lines, one of the two resulting siblings was arbitrarily selected for further analysis. The single-cell trajectories are from randomly selected sporulating (top panel) and nonsporulating (bottom panel) cells. The dotted vertical black line indicates that the spore became visible in the corresponding movie (top panel). (B) Movie S9 in the supplemental material (IDJ039) was used to create a histogram displaying the *rapA* promoter activity measured by GFP levels prior to spore formation (768 min of spore development). Sporulating cells (gray bars) show significantly ($P < 0.01$ by Student's t test) lower P_{rapA}-GFP levels than their nonsporulating siblings (black bars) analyzed at the same time in microcolony development.

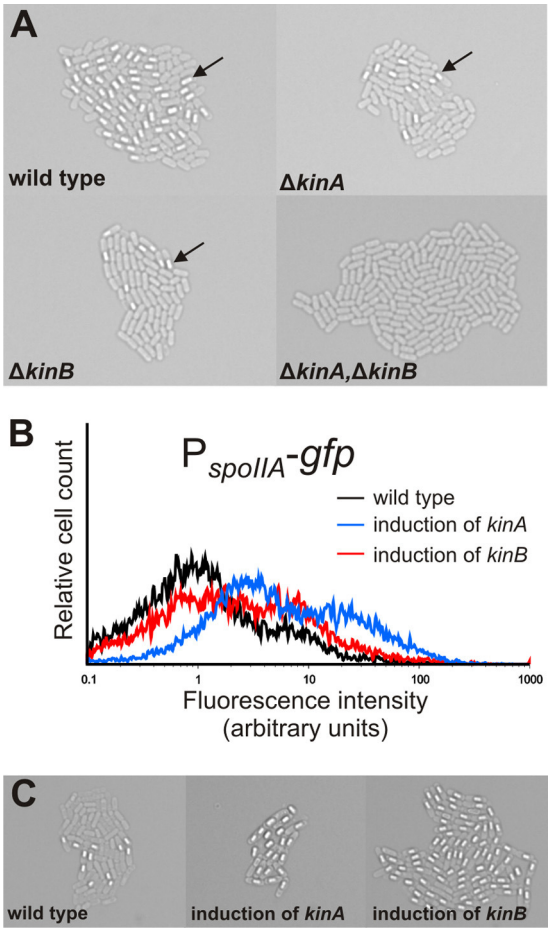

FIG. 5. The activity of the phosphorelay contributes to sporulation bistability. (A) Bright-field images of the wild-type strain and strains with mutations in KinA (IDJ035), KinB (IDJ036), or both kinases (IDJ037) after 24 h of microcolony development. Arrows point to spores in microcolonies of strains able to sporulate. (B) P_{spoIIA}-GFP activity of cells overproducing KinA (IDJ021) or KinB (IDJ022). Cells were grown in TY medium and induced with 100 μM IPTG in early exponential phase. At 5 h after induction, samples were analyzed by flow cytometry. (C) Spore development in microcolonies of strains with induced transcription of *kinA* (IDJ021) or *kinB* (IDJ022). Cells were grown as described in Materials and Methods and spotted on agarose-based chemically defined medium containing 100 μM IPTG.

ground, the effect of the single mutation in our genetic background (168 trp+) was not as dramatic as the effect of a *kinA* mutation on sporulation in the JH642 background (8).

Besides KinA and KinB, three other phosphorelay kinases (KinC, KinD, and KinE) that are not essential for spore formation have been identified (22, 44). To validate that KinA and KinB are the primary sporulation kinases in our experimental setup, a *kinA kinB* double mutant was constructed and analyzed by time-lapse microscopy. Consistent with previous reports (31, 39), spore formation was completely blocked in the *kinA kinB* double mutant (IDJ037) (Fig. 5A).

If cell-to-cell differences in phosphate input within the system result in bimodal *spoIIA* expression, overproduction of KinA or KinB might abolish sporulation heterogeneity. It was previously shown that artificial induction of *kinA* or *kinB* expression induces spore formation during exponential growth (15), indicating either that the kinases autophosphorylate readily and do not require a specific stationary-phase signal to switch to their kinase mode or that the basal level of kinase activity is sufficient to initiate sporulation under overproduction conditions. To investigate the effects of KinA or KinB overproduction on P_{spoIIA} activation, we cloned the genes coding for these kinases under the control of the strong IPTG-inducible *spank* promoter (see Materials and Methods) and transformed the corresponding DNA to strain IDJ007 (P_{spoIIA}-GFP), resulting in strains IDJ021 and IDJ022. To ensure that other stationary-phase signals leading to *spoIIA* activation were absent, cells were grown in rich TY medium and supplemented at early exponential phase with 100 μM IPTG to induce *kinA* or *kinB* expression. SDS-PAGE analysis of cell lysates clearly showed KinA overproduction at this IPTG concentration (data not shown). After 5 h of induction, cells were analyzed by flow cytometry. As shown in Fig. 5B, overproduction of KinA or KinB resulted in a shift of the GFP levels to the right, indicating that more cells activated the *spoIIA* promoter than in noninduced cultures. Interestingly, after 5 h of induction, not all cells have activated *spoIIA*, and a broad, almost bimodal *spoIIA* expression pattern is observed (Fig. 5B). This indicates that Spo0A~P does not reach the levels required to initiate sporulation in all cells, implying that the phosphorelay activity is not saturated under these KinA-overproducing conditions.

To confirm these results and directly assess the effect of KinA and KinB overproduction on sporulation heterogeneity in a clonal population originating from a single cell, the kinase overproduction strains were grown on agarose slides containing 100 μM IPTG and monitored by time-lapse microscopy. Under these conditions, overproduction of KinA or KinB resulted in premature spore formation (see Movies S11, S12, and S13 in the supplemental material). These results are consistent with the flow cytometry data showing an increased percentage of cells expressing high levels of P_{spoIIA}-GFP compared to the control. Importantly, upon KinA overproduction, microcolonies in which all cells formed spores (100% spores) were regularly found and sporulation heterogeneity was completely abolished (Fig. 5C; see Movie S11 in the supplemental material). In general, sporulation efficiency upon KinA overproduction was 92% ± 9% (mean ± standard deviation; data from six microcolonies, with 187 cells counted). Also in the case of KinB overproduction, most cells formed spores (82% ± 7%; data from seven microcolonies, with 356 cells counted), in sharp contrast to the case for wild-type microcolonies, where only approximately 14% (±10%; data from six microcolonies, with 630 cells counted) of the cells formed spores (Fig. 5C; see Movies S10 and S12 in the supplemental material). It is interesting to note that the total cell count (spores plus vegetative cells) per microcolony decreases with a decrease in sporulation heterogeneity (or an increase in sporulation efficiency). This can partly be explained by a block of vegetative cell division during sporulation (2), but nevertheless this indicates that sporulation heterogeneity gives a numerical (reproductive) advantage compared to homogenous sporulating populations.

Taken together, these data corroborate previous results showing the importance of the kinases in the initiation of sporulation (8, 15). Importantly, our results indicate that the phosphate input (through autophosphorylation of KinA or KinB), is a major determinant in the "decision" to sporulate or not.

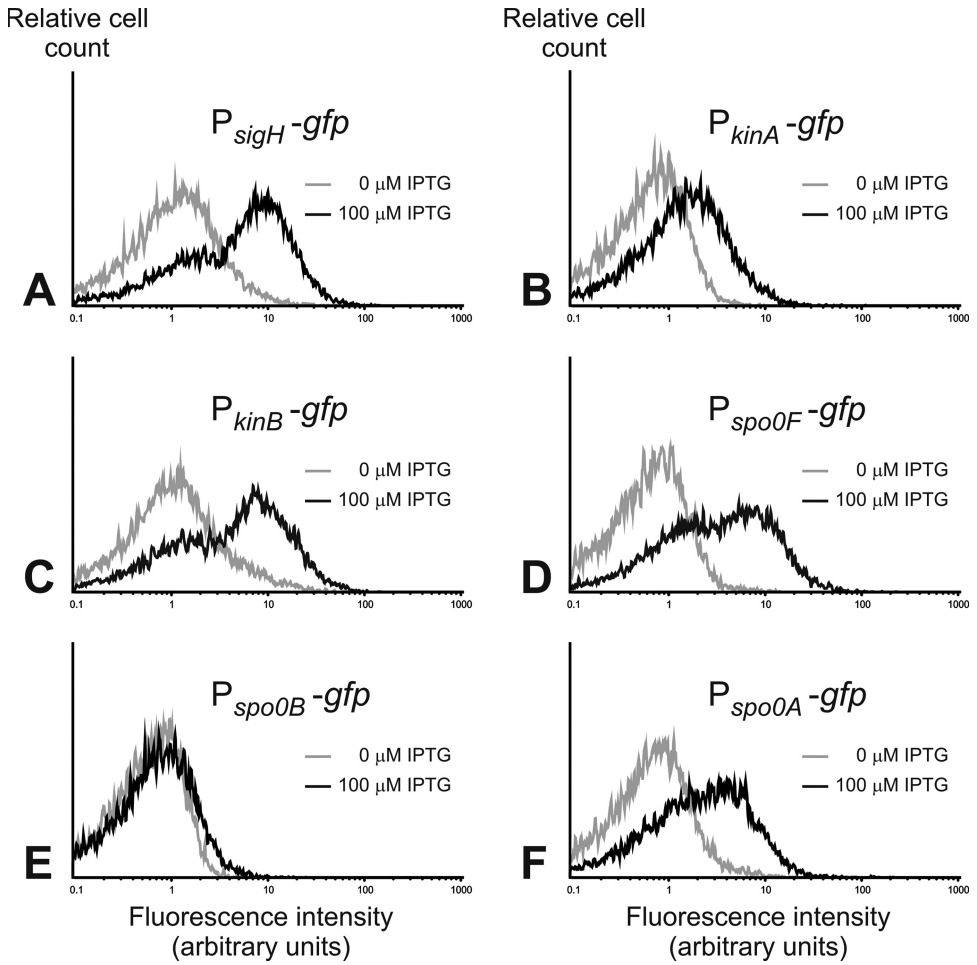

FIG. 6. Effects of KinA overproduction on phosphorelay promoter activity. Cells were grown in TY medium, and *kinA* expression was induced with 100 μM IPTG in early exponential phase. at 4 h after induction, the effect of *kinA* overexpression on the transcription of the phosphorelay promoters was analyzed by flow cytometry. (A) P_{sigH}-GFP (IDJ028); (B) P_{kinA}-GFP (IDJ029); (C) P_{kinB}-GFP (IDJ030); (D) P_{spo0F}-GFP (IDJ031); (E) P_{spo0B}-GFP (IDJ032); (F) P_{spo0A}-GFP (IDJ033).

KinA overproduction leads to upregulation of phosphorelay genes. The activity of the phosphorelay is determined by a mix of environmental and cell cycle cues (33). Thus, the question remains how these external signals are propagated through the network to initiate sporulation; how do high kinase levels manage to abolish sporulation heterogeneity? To test this, we examined the expression of P_{kinA}-GFP, P_{kinB}-GFP, P_{spo0F}-GFP, P_{spo0B}-GFP, P_{spo0A}-GFP, and P_{sigH}-GFP under conditions in which the phosphorelay was artificially charged (KinA overproduction) at a stage in which normally no stationary-phase signals are present (induction in TY medium at early exponential phase). Flow cytometry analysis showed that transcription of *kinA*, *kinB*, *spo0F*, *spo0A*, and *sigH* was upregulated by overproduction of KinA (Fig. 6). This indicates that the phosphate charge of the phosphorelay directly affects the transcription of the phosphorelay, which might be important for signal propagation. *spo0B* seems to be the exception, as its transcription is not under (indirect) positive control of KinA (Fig. 6E). This result might suggest that Spo0B is a limiting factor for the initiation of sporulation, but our experimental data show that this is unlikely (see Discussion and Fig. S5 in the supplemental material). Interestingly, at least for *sigH*, *kinB*, *spo0F*, and perhaps *spo0A*, a bimodal expression pattern arises upon KinA overproduction, again suggesting that upon KinA overproduction the phosphorelay is not fully saturated in all cells (Fig. 6).

A decreased phosphorelay flow results in fewer sporulating cells. Our data and previous work from other labs suggest that overproduction of KinA leads to an increase in the phosphate charge of the phosphorelay. In addition, it has been shown that overproduction of the Spo0E phosphatase reduces Spo0A~P levels and thus sporulation (30, 32) and that deletion of *spo0E* causes more cells to initiate sporulation (46). To further support the hypothesis that sporulation heterogeneity is regulated through the phosphate flow of the phosphorelay, we constructed a strain in which *spo0E* is under the control of P_{spank} (IDJ014). Genomic DNA of this strain was used to construct strain IDJ027, which also carries a xylose-inducible *kinA* construct (see Materials and Methods). Flow cytometry experiments were performed as described above for Fig. 5B, using 0.5% xylose for induction of *kinA* expression and 1 mM IPTG for induction of *spo0E* expression. Consistent with the data obtained with the IPTG-inducible *kinA* strain IDJ011, induc-

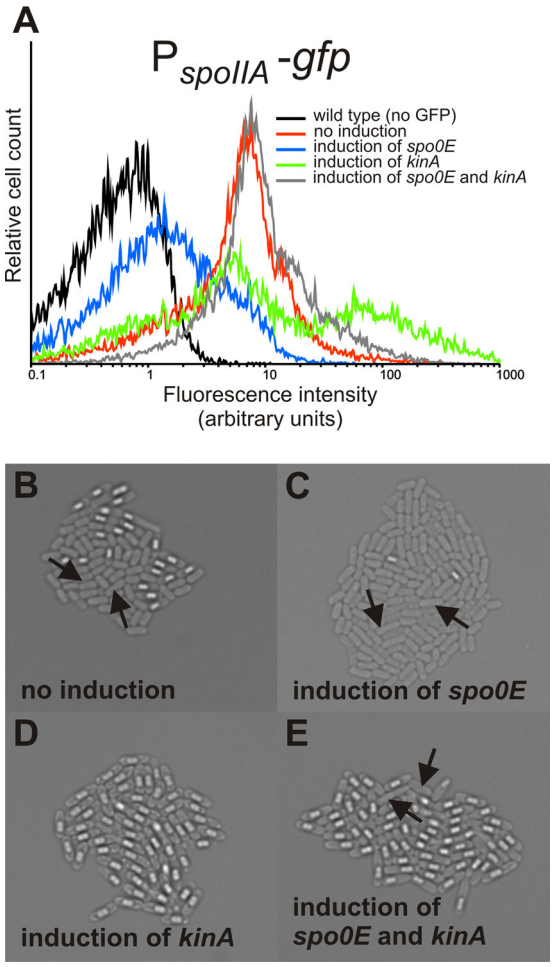

FIG. 7. *spo0E* overexpression negatively affects sporulation even in cells overproducing KinA. (A) P_{spoIIA}-GFP activity of cells overexpressing Spo0E or KinA. Strain IDJ027 (P_{spank}-*spo0E*, P_{xyl}-*kinA*, P_{spoIIA}-GFP) was grown in TY medium and induced at early exponential phase with 1 mM IPTG for Spo0E overproduction and/or with 0.5% xylose for KinA overproduction. A noninduced culture of the same strain and the wild type (no GFP expression) were used as controls. Samples were collected for flow cytometry at 4 h after induction. (B to E) Bright-field pictures of IDJ027 taken 24 h after microcolony development. Arrows point to a selection of nonsporulating cells. (B) No induction; (C) induction of *spo0E* expression with 1 mM IPTG; (D) induction of *kinA* expression with 0.5% xylose; (E) induction of *spo0E* expression with 1 mM IPTG and of *kinA* expression with 0.5% xylose.

tion of *kinA* expression from the xylose-inducible promoter (Fig. 7A, green line) resulted in a significant increase in GFP expression from the *spoIIA* promoter compared to that in the noninduced culture (Fig. 7A, red line). Upon overproduction of Spo0E, GFP expression from P_{spoIIA} was significantly reduced (blue line). Since Spo0E dephosphorylates Spo0A~P (30), these data support the idea that phosphate availability in the phosphorelay is the major determinant responsible for sporulation heterogeneity. Overproduction of both the kinase KinA and the phosphatase Spo0E (gray line) clarifies this even more, since the GFP expression pattern strongly resembles that of the noninduced control (red line). This indicates that the result of the increased KinA levels is diminished by the increased Spo0E levels. The same strain (IDJ027) was also used to examine the effect of decreased phosphate availability on sporulation heterogeneity by microscopy under the conditions used for time-lapse microscopy (Fig. 7B to E). The cells were grown on a 15% CDM agar pad supplemented with 0.5% xylose for *kinA* overexpression and 1 mM IPTG for *spo0E* overexpression, and snapshots of the microcolonies were taken after 24 h. As expected, sporulation heterogeneity was observed for the noninduced control (Fig. 7B) and heterogeneity was lost upon KinA overproduction (100% spore formation) (Fig. 7D). Conversely, upon overproduction of Spo0E, only a few cells reached the Spo0A~P levels required for spore formation, resulting in a significant reduction in sporulation efficiency (Fig. 7C). Consistent with the flow cytometry data, spore formation during Spo0E overproduction was restored by KinA overproduction (Fig. 7E).

A heterogeneous sporulation signal does not require positive feedback by SigH. The alternative sporulation sigma factor SigH activates transcription of *kinA*, *spo0F*, and *spo0A*, and indirectly its own transcription, but not that of *spo0B* (14, 35, 40). Thus, SigH constitutes a major positive-feedback loop in the sporulation network and might be important in determining whether a cell will sporulate or not. We showed that overproduction of KinA caused activation of *sigH* (Fig. 6A), suggesting that SigH plays a pivotal role in the propagation of the sporulation signal. To further explore the role of SigH, we deleted *sigH* and assessed the effect on heterogeneous *spoIIA* expression in microcolonies (IDJ034). Interestingly, while the *spoIIA* promoter is under SigH control (52), cells mutated in *sigH* still exhibited heterogeneous GFP expression (Fig. 8A; see Fig. S6 in the supplemental material). However, after 24 h of microcolony development, P_{spoIIA}-GFP expression in nonsporulating cells mutant in *sigH* was at least 2.5-fold lower than that in wild-type cells (Fig. 8A). Most likely the difference is even greater at earlier stages of microcolony development, when wild-type cells show the highest *spoIIA* expression. To test whether the heterogeneity was caused by Spo0A, we performed similar experiments with a P_{spoIIE}-GFP reporter strain in the *sigH* mutant background. *spoIIE* is also under the control of Spo0A but is not dependent on SigH for its expression (53). Although GFP expression from the *spoIIE* promoter in the *sigH* mutant background was even closer to the background fluorescence than GFP expression from P_{spoIIA} in this mutant, heterogeneity could still be observed (data not shown). Thus, both experiments indicate that heterochronic phosphorelay gene expression (in conjunction with the phosphorelay phosphate charge) is sufficient to create a heterogeneous sporulation signal and that the positive-feedback loop by SigH is probably required only as an amplifier to ensure that phosphate levels can reach the threshold required for actual spore formation. Nevertheless, we cannot exclude that SigH might be essential for other factors required for sporulation that were not tested with our studies.

If SigH merely amplifies the heterogeneous sporulation signal, an artificial increase of the phosphorelay phosphate charge might bypass the requirement of SigH for spore formation. To test this hypothesis, we overproduced KinA in a *sigH* mutant background (IDJ038). In this experimental setup, the activity of the phosphorelay is artificially charged in the absence of the SigH feedback loop. As shown in Fig. 8A, under these condi-

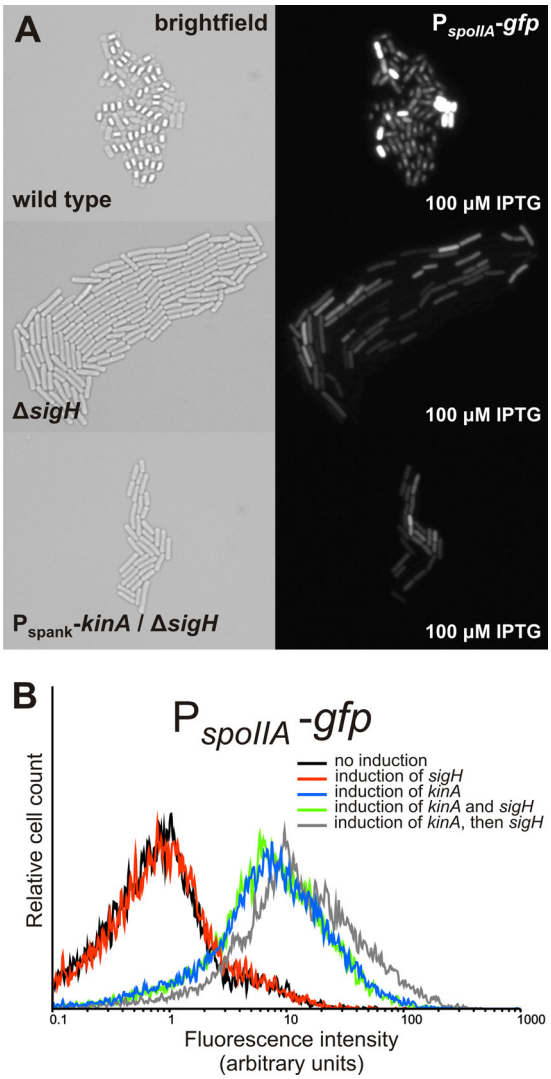

FIG. 8. Positive feedback by SigH is essential for spore formation. (A) Snapshots of wild-type (168 trp+), Δ*sigH* (IDJ020), and P_{spank}-*kinA* Δ*sigH* (IDJ037) microcolonies after 24 h. The agarose-containing medium was supplemented with 100 μM IPTG to overproduce KinA. This analysis shows that SigH is required for spore development (bright-field pictures, left panels) but not for bimodal expression of P_{spoIIA}-GFP (GFP pictures, right panels). (B) The temporal order of activation of SigH contributes to efficient initiation of sporulation. IDJ037 harboring the P_{spoIIA}-GFP reporter was grown in TY medium and induced with 1 mM IPTG for SigH overproduction and/or with 0.5% xylose for KinA overproduction at early exponential phase. Samples for flow cytometry were taken 4 h after the initial induction.

tions spores were also not formed, indicating that in the absence of the positive SigH feedback loop, Spo0A~P cannot reach levels high enough to initiate sporulation even if the phosphorelay is artificially charged.

If KinA overproduction activates initiation of sporulation only in the presence of an intact SigH feedback loop, the temporal separation between charging the phosphorelay and SigH expression might be important for sporulation heterogeneity. To test this, *kinA* was placed under the control of the xylose-inducible promoter P_{xyl} and *sigH* under the control of P_{spank}. Both constructs were stably integrated in the chromosome of the P_{spoIIA}-GFP reporter strain, and the resulting strain (IDJ026) was grown in the presence or absence of xylose and/or IPTG, respectively. Flow cytometry analysis of exponentially growing cells showed that P_{spoIIA}-GFP was not increased when *sigH* expression was artificially induced (in the presence of 1 mM IPTG) (Fig. 8B, red line). KinA overproduction (induced by 0.5% xylose) activated P_{spoIIA}-GFP, as expected (blue line). When both KinA and SigH were overproduced simultaneously, P_{spoIIA}-GFP was activated to a similar degree (green line) as under conditions in which only *kinA* was overexpressed. Strikingly, when KinA was overproduced first and SigH 1 h later, even more cells activated expression of the *spoIIA* operon (gray line). This might reflect the temporal order of processes within the system prior to heterogeneous initiation of sporulation. Upon external signal accumulation, the first process being operated is signal integration by buildup of the phosphate charge, followed by an increase in expression of the alternative sigma factor SigH, which in turn facilitates expression of *spo0A* and the sporulation-specific genes that require high levels of Spo0A~P.

DISCUSSION

Clonal populations of *Bacillus subtilis* bifurcate into sporulating and nonsporulating subpopulations. This phenotypic bistability, or sporulation heterogeneity, is proposed to have evolved as a "bet-hedging" strategy to ensure that part of the population is always prepared for uncertain future and rapidly changing environmental conditions (50). In contrast to competence development, the direct positive feedback on the main regulator Spo0A is not essential for sporulation heterogeneity (50). Since Spo0A phosphorylation and initiation of sporulation are controlled by a multicomponent phosphorelay, we hypothesized that sporulation heterogeneity can arise at two distinct levels: (i) variability in the expression of phosphorelay genes and/or (ii) variability in the overall net activity of the phosphorelay. Using single-cell analysis tools, we tested these predictions. In a previous study it was shown that there is a large cell-to-cell variability in low levels of *spo0A* expression during exponential growth (5). We now show that the other phosphorelay genes also show a large variability in gene expression (Fig. 2 and Fig. 3; see Fig. S1, S2, and S3 in the supplemental material). The simplest explanation for sporulation heterogeneity would be that at least one of the genes involved in activation of sporulation-specific genes is bimodally expressed. Surprisingly, all phosphorelay genes were expressed unimodally across the population (Fig. 2; see S1 in the supplemental material).

In line with previous genetic evidence (7, 16, 35, 41–43), histograms based on expression data of developing microcolonies showed that transcription of all phosphorelay genes, except for *spo0B*, increases as cells enter stationary growth (Fig. 2; see Fig. S1 in the supplemental material). If this temporal increase of transcription does not occur simultaneously in all cells, this could constitute another source for sporulation heterogeneity. In fact, single-cell expression traces showed that sporeformers in general activate transcription of *kinA*, *spo0F*, and *spo0A* earlier than nonsporulating cells within the same microcolony (Fig. 3; see Fig. S2 and S3 in the supplemental material). At this point we cannot distinguish whether the early

increase in *kinA* expression is caused by early activation of Spo0A or by KinA itself. This is currently under investigation in our laboratory.

Interestingly, the subpopulation of nonsporulating cells demonstrated an interesting pulsating gene expression pattern during microcolony development, which resembles recently reported fluctuations of *rapA* and *sda* gene expression (3, 48). It is tempting to speculate that cells undergo cell cycle-dependent regulation of the activity of Spo0A~P as a "bet-hedging" strategy to ensure that cells will sporulate only when the conditions are favorable again to bifurcate into sporulating and nonsporulating populations (50). Furthermore, cell cycle regulation of Spo0A~P is important to ensure correct chromosome copy number during sporulation (48).

Why do the nonsporulating cells show a relatively high level of transcription of the phosphorelay genes but do not initiate sporulation? To initiate sporulation, cells require high levels of Spo0A~P (14). Thus, although the phosphorelay gene expression of the nonsporulating subpopulation was shown to be high at late stages of microcolony development (Fig. 3; see Fig. S2 and S3 in the supplemental material), the net phosphate charge of the phosphorelay might be too low to initiate sporulation. In this respect it is interesting to note that transcription of the genes encoding the RapA and Spo0E phosphatases are activated by Spo0A~P as well (14, 32). Concomitantly with the phosphorelay genes, the expression of these negative regulators of phosphorelay activity is elevated, and Spo0A~P levels could be kept low although phosphorelay gene expression is relatively high. Overproduction of Spo0E resulted in a decrease in spore formation, a phenotype that could be bypassed by simultaneous overproduction of KinA (Fig. 7). In fact, using a double-labeled strain, we showed that nonsporulating cells with relatively high levels of *kinA* expression have significantly higher *rapA* transcription levels than their sporulating siblings (Fig. 4). RapA levels might, for instance, be influenced by growth rate, cell densities, and the nutritional status of the cell (3). This suggests that all cells have the predisposition to initiate sporulation but that only those able to reach a sufficiently high phosphorelay phosphate charge can enter the pathway.

It has been suggested that the phosphorelay kinases need a (biochemical) signal to initiate autophosphorylation, although the nature of this signal still remains elusive (24). Transcriptional activation (overproduction) of *kinA* or *kinB* is sufficient to increase Spo0A~P levels and initiate spore formation (15). Here we show that overproduction of *kinA* or *kinB* results in elevated levels of phosphorelay gene expression (Fig. 5 and 6), even under conditions in which no late-stationary-phase signals are present. Furthermore, KinA overproduction itself can abolish sporulation heterogeneity in microcolonies originating from a single cell, resulting in 100% sporulating cells (Fig. 5C). However, flow cytometry shows that overproduction of KinA does not abolish heterogeneity *per se* (Fig. 5B) but that it seems to reduce the time for individual cells within the heterogeneous population to commit to sporulation. These data indicate that autophosphorylation of the kinases occurs readily, possibly without the need of an external signal, in line with previous reports on the phosphorelay and other two-component systems in which overproduction of the signal kinase leads to signal-independent activation of the cognate response regulator (23). Whether the kinases are fully in their kinase mode during overproduction or whether the basal level of the kinase activity is sufficient to initiate sporulation under overproduction conditions is as yet unknown.

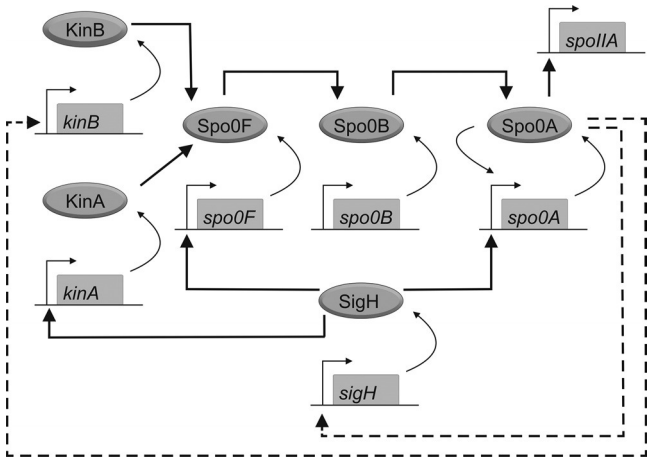

FIG. 9. Simplified transcriptional network of the *Bacillus subtilis* phosphorelay. Arrows and perpendiculars indicate positive and negative activities, respectively. Spo0A~P represses transcription of *abrB*, which is a repressor of *sigH* transcription. For simplicity, this indirect positive action of Spo0A on *sigH* is drawn as a direct action (dotted line). The same applies for the indirect positive regulation of *kinB* by Spo0A~P.

As mentioned before, phenotypic bistability can originate from positive feedback within the gene network. A major positive-feedback loop within the sporulation network is represented by the alternative sigma factor SigH. SigH was shown to activate transcription of *kinA*, *spo0F*, and *spo0A* (35). Furthermore, transcription of *sigH* is repressed by the unstable transcriptional repressor protein AbrB, whose transcription is repressed by low levels of Spo0A~P (14, 40). Thus, indirect transcriptional activation of *sigH* by Spo0A~P results in elevated levels of Spo0A~P, constituting a feedback loop (Fig. 9). Our data suggest that once the phosphorelay reaches a certain threshold phosphate charge, the SigH feedback loop kicks in, leading to even larger amounts of KinA and KinB (Fig. 6 and 8). This causes a rapid increase of Spo0A~P, ultimately resulting in activation of the unidirectional sporulation program. It is tempting to speculate that this built-in time delay requires a complex network topology with many feedback loops, as it is present within the phosphorelay (Fig. 9). Although the heterogeneous sporulation signal is established without the need for SigH, this positive-feedback loop might provide the system with more robustness.

Spo0B is special in the phosphorelay, as it is not under feedback control and might act to keep the system in the signal integration regimen (3). Initially, our data suggested that Spo0B might be a limiting factor for sporulation, since its expression was shown to be low and did not increase with time (Fig. 2F and 3F; see Fig. S1F, S2F, and S3F and Movie S6 in the supplemental material). These data are consistent with data from Ferrari et al., who estimated that only 50 to 100 Spo0B molecules are present in the cells (12). However, overproduction of Spo0B or Spo0F resulted in fewer cells expressing P_{spoIIA}-GFP (see Fig. S5 in the supplemental material). A likely explanation for this phenotype is that the ratio

of nonphosphorylated to phosphorylated phosphotransferase has increased. Since all the reactions in the phosphorelay are reversible (11) (Fig. 1), the effective concentration of the phosphate-bound phosphotransferase is reduced upon overproduction. This might reduce the speed of phosphotransfer to its cognate partner, subsequently resulting in reduced levels of Spo0A~P. This indicates that Spo0B is not a limiting factor for sporulation and that minor fluctuations in the levels of any of the phosphorelay components might rapidly be equilibrated between the components within the phosphorelay, making the system robust to posttranscriptional noise. On the other hand, the broad distribution of expression levels of some of the phosphorelay genes, such as *kinA*, *spo0F*, and *spo0A*, indicates that there is a large cell-to-cell variability in Spo0A~P levels which possibly originates from stochastic fluctuations that arise during the process of transcription.

In conclusion, our data strongly support a model in which sporulation heterogeneity originates from intercellular differences in phosphorelay phosphate availability regulated at both the transcriptional and posttranslational levels. While the Spo0A~P level increases over time, a large cell-to-cell variability in Spo0A~P exists, which may be caused by the cell cycle state, the metabolic activity of the cell, and/or noise in phosphorelay gene transcription. Here we use the term heterochronicity, since our data suggest that there is not only cell-to-cell variability in phosphorelay gene expression but also temporal variation between the development of cells. Thus, sporulation heterogeneity seems to originate from a combination of asynchrony and stochastic influences, such as transcriptional noise, but might also include specific metabolites or signaling molecules. Stochasticity of transcription might be an important regulatory mechanism for heterogeneity in phosphorelay gene expression, especially variations in kinase gene expression. Furthermore, our data suggest that successful entry into sporulation might require that all phosphorelay components reach a certain threshold concentration at the same time. If one or more components of the phosphorelay are below this threshold, sporulation cannot be initiated. This might also offer an explanation for the few cells that show a relatively early peak of *spo0F* transcription but do not go on to sporulate.

Not all *B. subtilis* cells sporulate at the same time, but with increasing time more cells sporulate. Whether cells initiate sporulation later might depend on the nutrients released by lysed cells, for instance, via the cannibalism route, but it also depends on the cell cycle state of the cell (17, 48). Here, we have followed sporulation only for a period of approximately 24 h. It would be interesting to see whether cells that have not sporulated during this period would divide again later and bifurcate into sporulating and nonsporulating cells. It is tempting to speculate that heterochronicity is an important feature of *B. subtilis* sporulation to allow for an optimal ratio of sporulating and vegetative cells.

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REFERENCES

1. **Asai, K., M. Fujita, F. Kawamura, H. Takahashi, Y. Kobayashi, and Y. Sadaie.** 1998. Restricted transcription from Sigma H or phosphorylated Spo0A dependent promoters in the temperature-sensitive secA341 mutant of *Bacillus subtilis*. Biosci. Biotechnol. Biochem. **62:**1707–1713.
2. **Ben-Yehuda, S., and R. Losick.** 2002. Asymmetric cell division in *B. subtilis* involves a spiral-like intermediate of the cytokinetic protein FtsZ. Cell **109:** 257–266.
3. **Bischofs, I. B., J. A. Hug, A. W. Liu, D. M. Wolf, and A. P. Arkin.** 2009. Complexity in bacterial cell-cell communication: quorum signal integration and subpopulation signaling in the *Bacillus subtilis* phosphorelay. Proc. Natl. Acad. Sci. U. S. A. **106:**6459–6464.
4. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64:**545–552.
5. **Castilla-Llorente, V., M. Salas, and W. J. Meijer.** 2008. *kinC/D*-mediated heterogeneous expression of *spo0A* during logarithmical growth in *Bacillus subtilis* is responsible for partial suppression of phi 29 development. Mol. Microbiol. **68:**1406–1417.
6. **Chary, V. K., E. I. Amaya, and P. J. Piggot.** 1997. Neomycin- and spectinomycin-resistance replacement vectors for *Bacillus subtilis*. FEMS Microbiol. Lett. **153:**135–139.
7. **Chibazakura, T., F. Kawamura, K. Asai, and H. Takahashi.** 1995. Effects of *spo0* mutations on *spo0A* promoter switching at the initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. **177:**4520–4523.
8. **Chung, J. D., G. Stephanopoulos, K. Ireton, and A. D. Grossman.** 1994. Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. J. Bacteriol. **176:**1977–1984.
9. **Dubnau, D., and R. Losick.** 2006. Bistability in bacteria. Mol. Microbiol. **61:**564–572.
10. **Eichenberger, P., M. Fujita, S. T. Jensen, E. M. Conlon, D. Z. Rudner, S. T. Wang, C. Ferguson, K. Haga, T. Sato, J. S. Liu, and R. Losick.** 2004. The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis*. PLoS Biol. **2:**e328.
11. **Fabret, C., V. A. Feher, and J. A. Hoch.** 1999. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. J. Bacteriol. **181:**1975–1983.
12. **Ferrari, F. A., K. Trach, and J. A. Hoch.** 1985. Sequence analysis of the *spo0B* locus reveals a polycistronic transcription unit. J. Bacteriol. **161:**556–562.
13. **Fujita, M.** 2000. Temporal and selective association of multiple sigma factors with RNA polymerase during sporulation in *Bacillus subtilis*. Genes Cells **5:**79–88.
14. **Fujita, M., J. E. Gonzalez-Pastor, and R. Losick.** 2005. High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. J. Bacteriol. **187:** 1357–1368.
15. **Fujita, M., and R. Losick.** 2005. Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. Genes Dev. **19:**2236–2244.
16. **Fujita, M., and Y. Sadaie.** 1998. Feedback loops involving Spo0A and AbrB in *in vitro* transcription of the genes involved in the initiation of sporulation in *Bacillus subtilis*. J. Biochem. **124:**98–104.
17. **Gonzalez-Pastor, J. E., E. C. Hobbs, and R. Losick.** 2003. Cannibalism by sporulating bacteria. Science **301:**510–513.
18. **Hartl, B., W. Wehrl, T. Wiegert, G. Homuth, and W. Schumann.** 2001. Development of a new integration site within the *Bacillus subtilis* chromosome and construction of compatible expression cassettes. J. Bacteriol. **183:** 2696–2699.
19. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for *Bacillus.* John Wiley & Sons Ltd., Chichester, United Kingdom.
20. **Hicks, K. A., and A. D. Grossman.** 1996. Altering the level and regulation of the major sigma subunit of RNA polymerase affects gene expression and development in *Bacillus subtilis*. Mol. Microbiol. **20:**201–212.
21. **Itaya, M., K. Kondo, and T. Tanaka.** 1989. A neomycin resistance gene cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. Nucleic Acids Res. **17:**4410.
22. **Jiang, M., W. Shao, M. Perego, and J. A. Hoch.** 2000. Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. Mol. Microbiol. **38:**535–542.
23. **Laub, M. T., and M. Goulian.** 2007. Specificity in two-component signal transduction pathways. Annu. Rev. Genet **41:**121–145.
24. **Lee, J., D. R. Tomchick, C. A. Brautigam, M. Machius, R. Kort, K. J. Hellingwerf, and K. H. Gardner.** 2008. Changes at the KinA PAS-A dimerization interface influence histidine kinase function. Biochemistry **47:**4051–4064.
25. **Lewis, P. J., and A. L. Marston.** 1999. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene **227:**101–110.
26. **Lewis, R. J., D. J. Scott, J. A. Brannigan, J. C. Ladds, M. A. Cervin, G. B. Spiegelman, J. G. Hoggett, I. Barak, and A. J. Wilkinson.** 2002. Dimer

formation and transcription activation in the sporulation response regulator Spo0A. J. Mol. Biol. **316:**235–245.
27. **Linn, T. G., A. L. Greenleaf, R. G. Shorenstein, and R. Losick.** 1973. Loss of the sigma activity of RNA polymerase of *Bacillus subtilis* during sporulation. Proc. Natl. Acad. Sci. U. S. A. **70:**1865–1869.
28. **Maamar, H., and D. Dubnau.** 2005. Bistability in the *Bacillus subtilis* K-state (competence) system requires a positive feedback loop. Mol. Microbiol. **56:**615–624.
29. **Molle, V., M. Fujita, S. T. Jensen, P. Eichenberger, J. E. Gonzalez-Pastor, J. S. Liu, and R. Losick.** 2003. The Spo0A regulon of Bacillus subtilis. Mol. Microbiol. **50:**1683–1701.
30. **Ohlsen, K. L., J. K. Grimsley, and J. A. Hoch.** 1994. Deactivation of the sporulation transcription factor Spo0A by the Spo0E protein phosphatase. Proc. Natl. Acad. Sci. U. S. A. **91:**1756–1760.
31. **Perego, M., S. P. Cole, D. Burbulys, K. Trach, and J. A. Hoch.** 1989. Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. J. Bacteriol. **171:**6187–6196.
32. **Perego, M., and J. A. Hoch.** 1991. Negative regulation of *Bacillus subtilis* sporulation by the *spo0E* gene product. J. Bacteriol. **173:**2514–2520.
33. **Perego, M., and J. A. Hoch.** 2002. Two-component systems, phosphorelays, and regulation of their activities by phosphatases, p. 473–481. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, DC.
34. **Piggot, P. J., and R. Losick.** 2002. Sporulation genes and intercompartmental regulation, p. 483–517. *In* A. L. Sonenshein, R. Losick, and J. A. Hoch (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, DC.
35. **Predich, M., G. Nair, and I. Smith.** 1992. *Bacillus subtilis* early sporulation genes *kinA*, *spo0F*, and *spo0A* are transcribed by the RNA polymerase containing sigma H. J. Bacteriol. **174:**2771–2778.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
37. **Smits, W. K., C. C. Eschevins, K. A. Susanna, S. Bron, O. P. Kuipers, and L. W. Hamoen.** 2005. Stripping *Bacillus*: ComK auto-stimulation is responsible for the bistable response in competence development. Mol. Microbiol. **56:**604–614.
38. **Smits, W. K., O. P. Kuipers, and J. W. Veening.** 2006. Phenotypic variation in bacteria: the role of feedback regulation. Nat. Rev. Microbiol. **4:**259–271.
39. **Spiegelman, G., H. B. Van, M. Perego, J. Day, K. Trach, and J. A. Hoch.** 1990. Structural alterations in the *Bacillus subtilis* Spo0A regulatory protein which suppress mutations at several *spo0* loci. J. Bacteriol. **172:**5011–5019.
40. **Strauch, M. A.** 1995. Delineation of AbrB-binding sites on the *Bacillus subtilis spo0H*, *kinB*, *ftsAZ*, and *pbpE* promoters and use of a derived homology to identify a previously unsuspected binding site in the *bsuB1* methylase promoter. J. Bacteriol. **177:**6999–7002.
41. **Strauch, M. A., K. A. Trach, J. Day, and J. A. Hoch.** 1992. Spo0A activates and represses its own synthesis by binding at its dual promoters. Biochimie **74:**619–626.
42. **Strauch, M. A., J. J. Wu, R. H. Jonas, and J. A. Hoch.** 1993. A positive feedback loop controls transcription of the *spoOF* gene, a component of the sporulation phosphorelay in *Bacillus subtilis*. Mol. Microbiol. **7:**967–974.
43. **Trach, K., J. W. Chapman, P. Piggot, D. LeCoq, and J. A. Hoch.** 1988. Complete sequence and transcriptional analysis of the *spo0F* region of the *Bacillus subtilis* chromosome. J. Bacteriol. **170:**4194–4208.
44. **Trach, K. A., and J. A. Hoch.** 1993. Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. Mol. Microbiol. **8:**69–79.
45. **Vasantha, N., and E. Freese.** 1980. Enzyme changes during *Bacillus subtilis* sporulation caused by deprivation of guanine nucleotides. J. Bacteriol. **144:** 1119–1125.
46. **Veening, J. W., L. W. Hamoen, and O. P. Kuipers.** 2005. Phosphatases modulate the bistable sporulation gene expression pattern in Bacillus subtilis. Mol. Microbiol. **56:**1481–1494.
47. **Veening, J. W., O. A. Igoshin, R. T. Eijlander, R. Nijland, L. W. Hamoen, and O. P. Kuipers.** 2008. Transient heterogeneity in extracellular protease production by Bacillus subtilis. Mol. Syst. Biol. **4:**184.
48. **Veening, J. W., H. Murray, and J. Errington.** 2009. A mechanism for cell cycle regulation of sporulation initiation in Bacillus subtilis. Genes Dev. **23:**1959–1970.
49. **Veening, J. W., W. K. Smits, and O. P. Kuipers.** 2008. Bistability, epigenetics, and bet-hedging in bacteria. Annu. Rev. Microbiol. **62:**193–210.
50. **Veening, J. W., E. J. Stewart, T. W. Berngruber, F. Taddei, O. P. Kuipers, and L. W. Hamoen.** 2008. Bet-hedging and epigenetic inheritance in bacterial cell development. Proc. Natl. Acad. Sci. U. S. A. **105:**4393–4398.
51. **Vellanoweth, R. L., and J. C. Rabinowitz.** 1992. The influence of ribosome-binding-site elements on translational efficiency in Bacillus subtilis and Escherichia coli in vivo. Mol. Microbiol. **6:**1105–1114.
52. **Wu, J. J., P. J. Piggot, K. M. Tatti, and C. P. Moran, Jr.** 1991. Transcription of the Bacillus subtilis spoIIA locus. Gene **101:**113–116.
53. **York, K., T. J. Kenney, S. Satola, C. P. Moran, Jr., H. Poth, and P. Youngman.** 1992. Spo0A controls the sigma A-dependent activation of Bacillus subtilis sporulation-specific transcription unit spoIIE. J. Bacteriol. **174:**2648–2658.